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Bioactive metabolites from the endophytic fungus *Alternaria alternata*

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ABSTRACT

Two altenuene derivatives (**1**–**2**) and one isocoumarin (**3**), together with six known compounds (**4**–**9**) were isolated from solid cultures of an endophytic fungus *Alternaria alternata*, obtained from the fresh branches of *Camellia sinensis*. Chiral analysis revealed the racemic nature of **1** and **2**, which were subsequently resolved into two pairs of enantiomers [(+)-**1** and (−)-**1**, (+)-**2** and (−)-**2**]. Structures of all the isolates were identified through spectroscopic data. Absolute configurations of the two pairs of enantiomers were determined by electronic circular dichroism (ECD) calculation and the chiral center of C-10 in **3** was deduced via $[Rh_2(OCOCF_3)_4]$-induced CD experiment. All the isolates were evaluated for their antimicrobial abilities against the pathogenic bacteria and fungi as well as cytotoxic activities against two human tumor cell lines. Compound **5** was the most active against *Bacillus subtilis* with MIC_{80} of 8.6 μg/ml, and compounds **1**–**3**, **6**–**7** and **9** exhibited moderate to weak inhibition towards the test pathogenic microorganism. Compound **4** showed mild cytotoxic activity against human osteosarcoma cells U2OS with IC_{50} of 28.3 μM.

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1. Introduction

Alternaria species have been identified as a prolific fungal source of new pharmacologically active metabolites such as steroids, terpenoids, pyrones, quinones, phenolics and nitrogen-containing compounds, which were suitable for specific medicinal or agricultural applications [1]. Dibenzopyranones with polyketide origin, such as alternariols and altenuenes were typical isolates from the genus *Alternaria*. These dibenzopyranone derivatives were examined to have various biological activities and functions, mainly including cytotoxicity [2,3] and antimicrobial [4,5] properties. During the investigation of bioactive natural products from plant endophytes, our attention was drawn to a strain of *Alternaria* sp., whose crude extract displayed potent inhibitory abilities against *Staphylococcus aureus* and *Bacillus subtilis* with inhibition zones of 19.5 and 25.3 mm respectively in the preliminary agar diffusion screening (37.7 mm of the positive control penicillin). Therefore, a systematic chemical study was performed and resulted in the isolation of altenuene-2-acetoxy ester (**1**), altenuene-3-acetoxy ester (**2**) and a new isocoumarin (+)-(10*R*)-7-hydroxy-3-(2-hydroxy-propyl)-5,6-dimethyl-isochromen-1-one (**3**), along with six known dibenzopyranone derivatives, alternariol 9-methyl ether (**4**) [5], alternariol (**5**) [5], phialophoriol (**6**) [6], altenuene (**7**) [7], 5′-epialtenuene (**8**) [8] and alterlactone (**9**) [2]. Chiral analysis of **1** and **2** revealed the presence of racemic mixture, and subsequent racemic resolution resolved into two pairs of enantiomers (+)-**1** [(+)-(2*S*, 3*S*, 4a*S*)-altenuene-2-acetoxy ester], (−)-**1** [(−)-(2*R*, 3*R*, 4a*R*)-altenuene-2-acetoxy ester], (+)-**2** [(+)-(2*S*, 3*S*, 4a*S*)-altenuene-3-acetoxy ester] and (−)-**2** [(−)-(2*R*, 3*R*, 4a*R*)-altenuene-3-acetoxy ester]. Details of the isolation, structure elucidation and bioactive evaluation of these compounds are discussed below.

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2. Experimental

2.1. General

Optical rotations were determined on a JASCO P-1020 polarimeter at room temperature. CD spectra were performed on a JASCO J-810 spectrometer. UV spectra were recorded on a Shimadzu UV-2501 spectrophotometer. IR spectra were measured by a Bruker Tensor 27 spectrometer. 1D and 2D NMR were carried out on a Bruker Avance III NMR (^{1}H: 500 MHz, ^{13}C: 125 MHz) instrument at 300 K, with TMS as internal standard. ESIMS was obtained on an Agilent 1100 Series LC/MSD ion-trap mass spectrometer and HRESIMS was recorded on an Agilent 6520B UPLC-Q-TOF mass spectrometer, respectively. Chiral HPLC was done with a Daicel Chiralpak AD-H column (250 × 4.6 mm, 5 μm). Preparative HPLC was performed by a Shimadzu LC-10A equipped with a Shim-pack RP-C_{18} column (20 × 200 mm) and a Shimadzu SPD-20A detector. Column chromatography (CC) was performed with silica gel and Sephadex LH-20. All the solvents used were of analytical grade.

2.2. Fungal material

The title strain was isolated from the branches of *Camellia sinensis*, collected from the suburb of Nanjing, Jiangsu Province, People's Republic of China, in October 2012. The culture was grown on potato dextrose agar (PDA) and distinguished morphologically as *Alternaria* sp., which was further reinforced by 18S rDNA sequence with a 100% identity to *Alternaria alternata*, conducted by the fungal identification service (Shanghai Majorbio Bio-pharm Technology).

The fungal strain was cultivated on PDA medium at 28 °C until sufficient growth was observed (about 5 days). Then plugs of agar supporting mycelia growth were cut into small pieces and transferred aseptically into a 1000 ml Erlenmeyer flask containing 300 ml potato dextrose broth, and incubated on a rotary shaker at 180 rpm for 7 days to prepare the seed culture. Fermentation was carried out in ten 500 ml Erlenmeyer flasks, each containing 80 g of rice and soaked with 120 ml distilled water overnight before autoclaving. Each flask was inoculated with 10 ml seed liquid, and cultivated under stationary conditions at 28 °C for 30 days.

2.3. Extraction and isolation

The fermented substrate was saturated with methanol and filtrated. After removal of the solvent under reduced pressure, the crude extract (8.6 g) was subjected to a silica gel column, eluted with a gradient of petroleum ether and acetone mixture from 20:1 to 1:1 (v/v) to generate six fractions (A–F). Fraction C (1.6 g) gave the main compounds **4** (19.0 mg) and **5** (9.5 mg) after Sephadex LH-20 CC (CH_2Cl_2–MeOH, 1:1). Fraction E (1.3 g) was chromatographed over silica gel CC (CH_2Cl_2–Acetone, 25:1, 15:1, 5:1, v/v) and further purified by preparative HPLC using MeOH–H_2O (60:40, 10 ml/min) to yield **1** (5.7 mg) and **2** (3.5 mg). Fraction D (2.0 g) was applied onto a silica gel CC, and eluted with CH_2Cl_2–MeOH (20:1, 10:1, 5:1, v/v) to acquire five major subfractions (D1–D5). Subfraction D2 (0.7 g) gave compound **6** (2.7 mg) after CC on Sephadex LH-20 (MeOH). Subfraction D3 (0.9 g) displayed in the same way as subfraction D2, and further purified by preparative HPLC to afford compounds **7** (1.0 mg) and **8** (1.1 mg) with MeOH–H_2O (65:35, 10 ml/min) as mobile phase. Compound **3** (2.5 mg) was obtained from fraction D4 (0.2 g) by preparative HPLC using methanol in water (60:40, 10 ml/min), and compound **9** (2.0 mg) was purified from fraction F (0.2 g) eluting with MeOH–H_2O (55:45, 10 ml/min). Compounds (+)-**1** (3.7 mg) and (−)-**1** (0.9 mg) were separated from **1** by a chiral column using *n*-hexane–isopropanol (90:10, 0.80 ml/min), and compounds (+)-**2** (1.5 mg) and (−)-**2** (1.2 mg) were isolated from **2** (*n*-hexane/isopropanol, 80:20, 0.75 ml/min) by the similar procedure of **1**.

Compound **1**: white solid; UV (MeOH) λ_{max} (logε): 193 (3.86), 200 (3.91), 241 (4.27), 278 (3.78), 319 (3.53) nm; IR (KBr) ν_{max}: 3444, 2922, 2349, 1627, 1384, 1263, 1163, 1117 cm^{-1}; ESIMS positive *m/z*: 335 $[M + H]^+$; HRESIMS *m/z*: 335.1124 $[M + H]^+$ (calcd for $C_{17}H_{19}O_7$, 335.1125); and ^{1}H and ^{13}C NMR data ($CDCl_3$), see Table 1.

(+)-**1** $[\alpha]_D^{25}$ + 13.3 (*c* 0.17, MeOH); CD (2.0 × 10^{-4}, MeOH) λ_{max} nm ($\Delta\varepsilon$) 241 (−7.43), 280 (+4.84).
(−)-**1** $[\alpha]_D^{25}$ − 13.3 (*c* 0.11, MeOH); CD (1.0 × 10^{-4}, MeOH) λ_{max} nm ($\Delta\varepsilon$) 241 (+3.67), 280 (−2.49).

Compound **2**: white solid; UV (MeOH) λ_{max} (logε): 194 (3.81), 200 (3.86), 240 (4.19), 277 (3.72), 317 (3.46) nm; IR (KBr) ν_{max}: 3450, 2923, 2852, 1642, 1384, 1163 cm^{-1}; ESIMS positive *m/z*: 335 $[M + H]^+$; HRESIMS *m/z*: 335.1127 $[M + H]^+$ (calcd for $C_{17}H_{19}O_7$, 335.1125); and ^{1}H and ^{13}C NMR data ($CDCl_3$) see Table 1.

(+)-**2** $[\alpha]_D^{25}$ + 11.6 (*c* 0.11, MeOH); CD (2.0 × 10^{-4}, MeOH) λ_{max} nm ($\Delta\varepsilon$) 241 (−2.34), 280 (+1.45).
(−)-**2** $[\alpha]_D^{25}$ − 11.6 (*c* 0.12, MeOH); CD (2.0 × 10^{-4}, MeOH) λ_{max} nm ($\Delta\varepsilon$) 241 (+2.35), 280 (−1.46).

Compound **3**: white solid; $[\alpha]_D^{25}$ + 35.3 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε): 213 (3.88), 244 (3.65), 252 (3.61), 292 (3.51) nm; CD (2 × 10^{-4}, MeOH) λ_{max} nm ($\Delta\varepsilon$) 365 (+3.67), 426 (−2.92); IR (KBr) ν_{max}: 3448, 2927, 1645, 1550, 1463, 1384, 1270, 1115 cm^{-1}; ESIMS positive *m/z*: 249 $[M + H]^+$; HRESIMS *m/z*: 249.1120 $[M + H]^+$ ($C_{14}H_{17}O_4$, calcd for 249.1121); and ^{1}H and ^{13}C NMR data (Acetone-d_6) see Table 1.

2.4. Absolute configuration of the secondary alcohol in 3

A solid $[Rh_2(OCOCF_3)_4]$ (2.0 mg) was added into compound **3** (0.7 mg) dissolving in CH_2Cl_2. After mixing, the first CD spectrum was recorded at once, and the time evolution was monitored until stationary (15 min). The inherent CD spectrum was subtracted. The metal complexes gave an induced CD spectrum, in which the sign of the E band around 350 nm was correlated to the absolute configuration of **3** [9,10].

2.5. Biological assays

The antibacterial activities against Gram-positive *S. aureus* ATCC 25923 (*S. aureus*), *B. subtilis* ATCC 6633 (*B. subtilis*) and Gram-negative *Escherichia coli* ATCC 25922 (*E. coli*) were initially performed by disk diffusion assay [11]. Each paper disk (6 mm diameter) permeating with 10 μl of test sample

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